

microscopy observation of matrix proteins in vessels shows a morphology different from what we observed when using fresh tissue.<sup>36</sup> Fixed samples show smaller, curly matrix fibers whereas in fresh tissue thick, elongated, straight fibers can be observed. This morphological observation has important consequences when considering that, starting only from the physiological ordered structure, we have been able to detect the modification induced by minimally modified LDL, resulting in curly and broken fibers.<sup>30</sup>

As an example of obtainable images, in Fig. 5 we report a series of images taken by scanning adjacent frames, from the lumen (top left) to the exterior (bottom right) of a portion of an aorta cross-section ring.

<sup>36</sup> A. A. Young, I. J. LeGrice, M. A. Young, and B. H. Smaill, *J. Microsc.* **192**, 139 (1998).

## [15] Photonics for Biologists

By IAN PARKER

Volumes 360 and 361 of *Methods in Enzymology* illustrate the wide array of powerful biophotonic approaches that have been developed to address biological problems. However, most biologists have little background in fields such as optics and laser technology, and may be daunted by the task of trying to understand how these techniques work or how to develop and construct photonic instrumentation for their own applications. This article is directed at such an audience and is divided into two main sections, which aim to provide (1) a primer in basic optics and functioning of the light microscope and (2) practical guidance on the functioning of photonic components and construction of complete biophotonic instrumentation.

### Introduction to Basics of Optics

#### *Classic Optics*

This section covers only the basics of optics and microscopy; but it is surprising how far a rudimentary knowledge will take you. For further reading, see Refs. 1–4, and the Web resources listed in the Appendix. The catalogs of many of the

<sup>1</sup> M. Abramowitz, "Optics: A Primer." Olympus America, New York, 1984.

<sup>2</sup> E. Hecht and A. Zajac, "Optics." Addison-Wesley, Reading, MA, 1974.

<sup>3</sup> M. C. Gupta, "Handbook of Photonics." CRC Press, Boca Raton, FL, 1997.

<sup>4</sup> F. A. Jenkins and H. E. White, "Fundamentals of Optics." McGraw-Hill, New York, 1976.

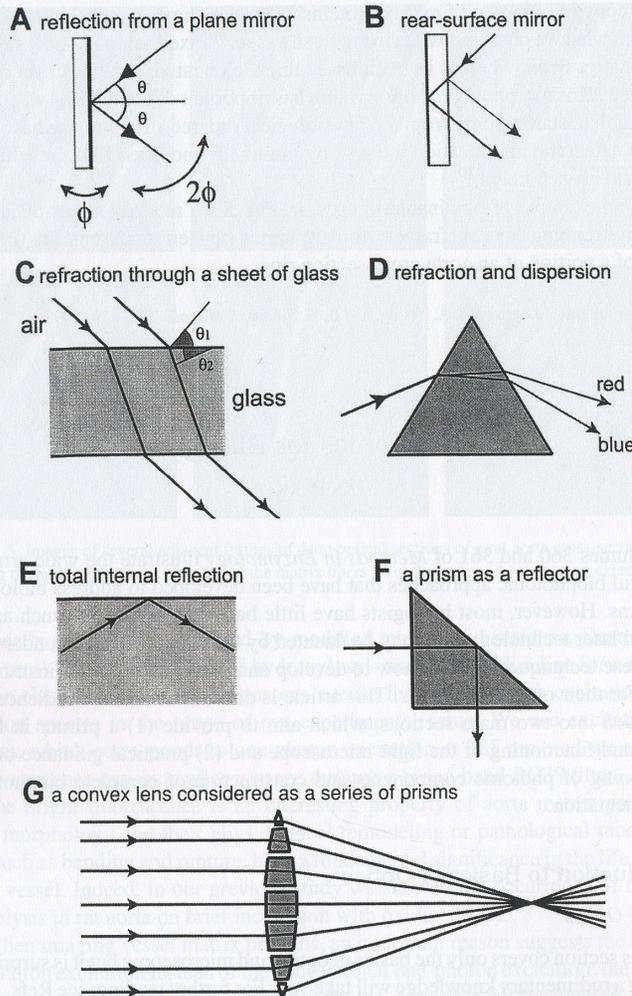


FIG. 1. Reflection and refraction at plane surfaces. (A) Reflection from a front-surface mirror. The incident and reflected rays subtend the same angle with regard to the mirror. If the mirror is rotated, light will move through an angle twice as great. (B) Rear-surface mirrors result in reflection both from the mirrored surface and, more faintly, from the front of the glass. (C) Light rays striking an air-glass interface at an angle are bent because of the change difference in refractive index ( $n$ ):  $n_{\text{air}} \sin \theta_1 = n_{\text{glass}} \sin \theta_2$ . An inverse change of angle results when the light exits the far side of a sheet of glass

major optical vendors are also a valuable source of practical information on basic optics, as well as on the specifics of particular products.

*Nature of Light.* Light is a tiny part of the electromagnetic spectrum, extending from wavelengths of about 200 to 2000 nm. Within this range the human eye can see only from about 400 nm (violet) to 700 nm (deep red); a mere 2-fold span in wavelengths as compared, for example, with the  $> 10^7$ -fold span of radio waves. The speed of light in vacuum or air is about  $3 \times 10^8 \text{ m s}^{-1}$ , which seems inconceivably fast until you consider that lasers can generate pulses of light as brief as 10 fs, so that each pulse has a length of only a few microns. Light has a dual nature, behaving as both a wave and as particles (photons). The energy of a photon increases as the reciprocal of the wavelength, so violet photons at 400 nm have twice the energy of infrared photons at 800 nm. A general rule seems to be that light will behave in whichever way presents the most obstacles for you. For example, when trying to image very small objects, the wavelength of light sets a limit to resolution, whereas when trying to detect very dim signals the discrete nature of individual photons introduces noise. In the latter regard, the statistical variance of the number of photons arriving per unit time increases as the square root of the mean photon flux, so that the signal-to-noise ratio improves proportional to the square root of light intensity.

*Reflection at Plane Surfaces.* Light incident on a plane mirror is reflected at an angle equal to the angle of incidence (Snell's law; Fig. 1A). If the mirror is rotated, the reflected light will rotate through an angle twice that of the mirror (an important point to remember when constructing laser scan systems). Optical mirrors are commonly made by depositing a reflective metal coating (silver or aluminum) onto the front of a flat glass substrate. However, reflection will occur at any interface between materials of different refractive index, to an extent proportional to the difference in indices (typically 4% for a glass-air interface). Thus, rear-surface mirrors should never be used, as reflection at the front glass surface causes a faint "ghost" reflection offset from the bright reflection from the rear-mirrored surface (Fig. 1B).

*Refraction.* The speed of light is fastest in a vacuum, and slows to varying extents in different materials. The ratio of the velocity of light in a vacuum to the velocity in a material is referred to as the refractive index ( $n$ ) of that material.

with parallel sides, resulting in a lateral translation of the light beam without any net change in angle. (D) Glass components with nonparallel sides, such as prisms, cause deviation in the angle of light rays. Because the refractive index of glass varies with wavelength, shorter wavelengths are deviated more than longer wavelengths (dispersion). (E) If light rays within glass strike an air-glass interface at a sufficiently shallow angle, they are reflected back into the glass without loss. (F) Use of total internal reflection within a right-angle prism to reflect a light beam. (G) A convex lens considered as a series of prisms.

Some examples include the following:

Medium	Refractive index
Vacuum	1
Air	1.0003
Water	1.333
Living cells	1.36
Optical glass	1.52
Diamond	2.42

When a beam of light passes an interface between materials of differing refractive index it bends (Fig. 1C), traveling at a steeper angle to the normal in medium of higher refractive index. (Imagine a line of people running at an angle along a beach and into the sea. They cannot run as fast when in the sea, so the angle of the line as a whole steepens.) For a sheet of glass with parallel sides the reverse process takes place at the glass-air interface on the opposite side. Thus, light traveling through a sheet of glass is not changed in direction, but is translated sideways, to an extent that depends on the angle of incidence and the thickness of glass (Fig. 1C).

In optical components such as prisms, the sides of which are not parallel, a net change in direction of light rays does result (Fig. 1D). Furthermore, because the refractive index of glass varies with wavelength, short wavelengths are bent more than longer wavelengths (dispersion).

**Total Internal Reflection.** Light rays traveling in a material of high refractive index (e.g., glass) are bent to a shallower angle when passing through an interface to a material of lower refractive index (e.g., air). If the angle of incidence is brought to a critical angle the refracted rays follow the surface of the interface, and at yet shallower angles of incidence the light is reflected back into the glass. This process is without loss (total internal reflection; Fig. 1E). The refractive index of glass is sufficiently high that a right-angle prism can thus be used as an efficient reflector (Fig. 1F), although there will still be losses at the air-glass interfaces and in transmission through the prism.

**Simple Lenses.** Imagine a series of prisms of progressively increasing steepness, arranged such that parallel rays of light striking each prism are bent so that they all pass through the same point (Fig. 1G). A convex lens with spherical surfaces provides a close approximation to this situation, and will focus parallel rays of light to a spot at a distance of one focal length behind the lens (Fig. 2A). The focal length becomes shorter for lenses with a smaller radius of curvature and higher refractive index. A lens with concave surfaces has a negative focal length, and diverges parallel rays such that they appear to have arisen from a point one focal length in front of the lens (Fig. 2B).

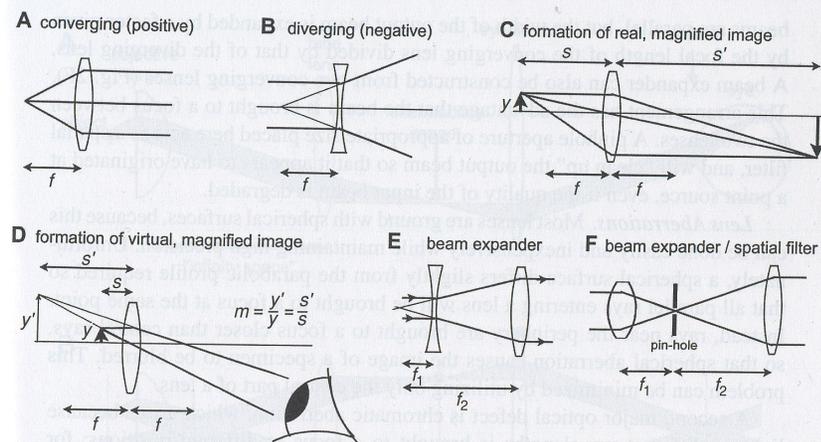


FIG. 2. Converging and diverging lenses. (A) A converging (positive) lens focuses a parallel beam of light to a spot at a distance one focal length ( $f$ ) from the lens. (B) A diverging (negative) lens diverges a parallel beam of light so that its rays appear to have originated from a point one focal length from the lens. (C) An object placed further than one focal length from a converging lens will form a real image on the other side of the lens. The magnification  $m$  (image height  $y'$ /object height  $y$ ) is given by  $s'/s$ . (D) An object placed closer than the focal length will not form a real image, but a virtual, magnified image can be viewed by the eye. This appears as right way up, behind the lens at the position marked by the thin arrow. Magnification is again given by  $s'/s$ . (E) A diverging and converging lens arranged as a beam expander. An incident beam of parallel light is expanded to form a parallel beam of greater diameter. The degree of expansion is given by  $f_2/f_1$  ( $f_2$  = focal length of converging lens,  $f_1$  = focal length of diverging lens). (F) A beam expander made from two converging lenses of different focal length. The first (short focal length) focuses an incident laser beam to a spot, which is then recollimated by the second lens. A pinhole aperture can be placed at the focal point to act as a spatial filter and "clean up" the exiting beam.

An object placed anywhere in front of a convex lens at distances between one focal length and infinity will form a real image (i.e., one that can be projected onto a piece of card) on the opposite side of the lens. The degree of magnification depends on the respective object and image distances, as shown in Fig. 2C. If the object is placed within one focal length of the lens a real image is not formed (because light rays emerging from the lens are still diverging). However, a virtual image can be viewed by the eye (which focuses the light rays to form a real image on the retina), and appears upright with a magnification as shown in Fig. 2D.

A diverging lens and converging lens can be arranged as in Fig. 2E to form a beam expander, useful for expanding a laser beam before introducing it into a microscope so as to fill the aperture of the objective lens. Both the input and output

beams are parallel, but the width of the output beam is expanded by a factor given by the focal length of the converging lens divided by that of the diverging lens. A beam expander can also be constructed from two converging lenses (Fig. 2F). This arrangement has the advantage that the beam is brought to a focus between the two lenses. A pinhole aperture of appropriate size placed here acts as a spatial filter, and will "clean up" the output beam so that it appears to have originated at a point source, even if the quality of the input beam is degraded.

**Lens Aberrations.** Most lenses are ground with spherical surfaces, because this can be done easily and inexpensively while maintaining high precision. Unfortunately, a spherical surface differs slightly from the parabolic profile required so that all parallel rays entering a lens will be brought to a focus at the same point. Instead, rays near the periphery are brought to a focus closer than central rays, so that spherical aberration causes the image of a specimen to be blurred. This problem can be minimized by utilizing only the central part of a lens.

A second major optical defect is chromatic aberration, which arises because light of different wavelengths is brought to a focus at different positions: for example, blue light is refracted to a greater extent than red, so that blue light will be focused to a spot closer to the lens than red light. For applications involving monochromatic laser beams chromatic aberration is not an issue, because only a single wavelength is present.

Both spherical and chromatic aberrations can be effectively corrected by use of more complex lenses employing multiple elements with materials of different refractive index and dispersion, or aspheric glass elements. The design of such lenses is complex. If aberrations from a simple lens are too great to be tolerated, the easiest solution is to purchase a microscope objective or other well-corrected compound lens.

### Optical Microscope

Most techniques in optical biology involve interfacing an external optical system to a microscope. A good place to begin, therefore, is to describe the optical functioning of a "generic" modern commercial microscope, and consider how this may be adapted to uses beyond those envisaged by the manufacturers. For further readings an excellent starting point is the review by M. W. Davidson and M. Abramowitz (<http://micro.magnet.fsu.edu/primer/opticalmicroscopy.html>); and see also Refs. 5–7.

<sup>5</sup> S. Bradbury and B. Bracegirdle, "Introduction to Light Microscopy." BIOS Scientific Publishers, Oxford, 1998.

<sup>6</sup> M. Abramowitz, "Fluorescence Microscopy: The Essentials." Olympus America, New York, 1993.

<sup>7</sup> B. Herman and J. J. Ledmasters, eds., "Optical Microscopy: Emerging Methods and Applications." Academic Press, New York, 1993.

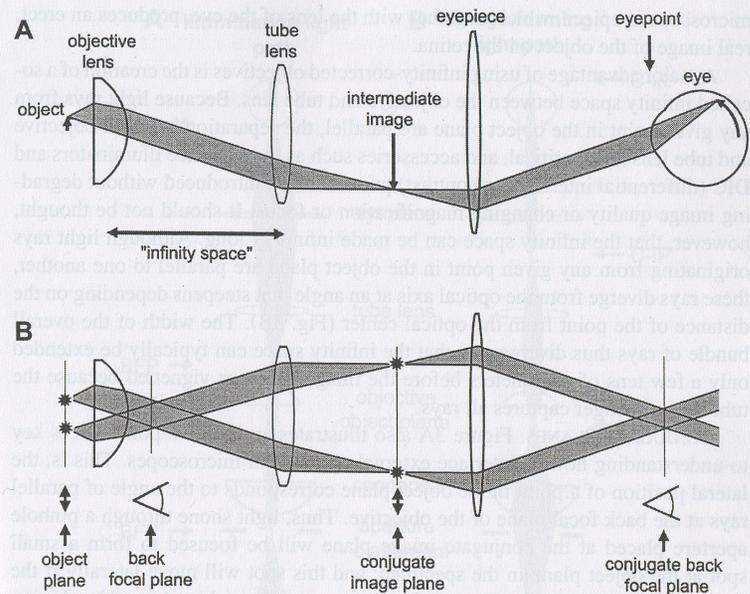


FIG. 3. Image ray paths within an infinity-corrected microscope. (A) Ray path illustrating the formation of a magnified virtual image. (B) Ray paths from two, laterally separated points in the specimen. Note that the lateral position at the object plane corresponds to the angle of parallel light rays at the back focal plane of the objective, and that these planes are reimaged at further points in the microscope.

### Fundamentals of Image Formation by Microscope

**RAY PATH IN INFINITY-CORRECTED MICROSCOPE.** All the major microscope manufacturers have now adopted infinity-corrected optics, in which the optical train within the microscope follows the scheme diagrammed in Fig. 3A. When correctly focused for optimal correction of aberrations, light from a point source in the specimen, such as a tiny fluorescent bead, is focused by the objective lens to form parallel beams of light (i.e., an image is formed only infinitely far behind the lens). Thus, unlike earlier designs in which objectives formed a real image at a fixed distance within the microscope tube (usually 160 mm), infinity-corrected lenses do not directly form an image. Instead, a further "tube" lens, located in the microscope body or ocular head, is used to form an intermediate image. This lies at a conjugate image plane, at the position of the eyepiece graticule. Note that this is a real image. It can be projected on a piece of card or captured by a camera body placed at the conjugate image plane. The image is then further magnified by the

microscope eyepiece, which, together with the lens of the eye, produces an erect, real image of the object on the retina.

A major advantage of using infinity-corrected objectives is the creation of a so-called infinity space between the objective and tube lens. Because light rays from any given point in the object plane are parallel, the separation between objective and tube lens is not critical, and accessories such as fluorescence illuminators and DIC (differential interference contrast) prisms can be introduced without degrading image quality or changing magnification or focus. It should not be thought, however, that the infinity space can be made infinitely long. Although light rays originating from any given point in the object plane are parallel to one another, these rays diverge from the optical axis at an angle that steepens depending on the distance of the point from the optical center (Fig. 3B). The width of the overall bundle of rays thus diverges, so that the infinity space can typically be extended only a few tens of centimeters before the image becomes vignetted because the tube lens no longer captures all rays.

**CONJUGATE PLANES.** Figure 3A also illustrates an intuitive point that is key to understanding how to interface external optics with microscopes. This is, the lateral position of a point in the object plane corresponds to the angle of parallel rays at the back focal plane of the objective. Thus, light shone through a pinhole aperture placed at the conjugate image plane will be focused to form a small spot at the object plane in the specimen; and this spot will move laterally if the pinhole is moved laterally. Conversely, the same result can be achieved by shining a parallel beam of light into the eyepiece (after removing the pinhole). This will again result in a focused spot at the object plane, but the lateral position of the spot can now be changed by varying the angle of the rays at the conjugate back focal plane (corresponding to the eyepoint, or position where the pupil of the eye would normally be located).

This concept is further illustrated in Fig. 4, showing the paths of illuminating light rays (A) and imaging light rays (B) within a microscope set up for bright-field illumination. A point in sharp focus in the specimen (i.e., at the object plane) is reimaged at several conjugate image planes, which lie at the positions of the retina of the observer, the eyepiece graticule, and the field diaphragm of the condenser (Fig. 4B). Thus, the object, the field diaphragm, and the graticule will all appear simultaneously in focus to the observer. On the other hand, the image field of the microscope should appear uniformly illuminated, without any visible structure from the lamp filament. This arrangement is known as Kohler illumination, and can be readily illustrated by looking at a flashlamp through a magnifying glass while moving the magnifying glass back and forth until the lens appears uniformly illuminated. At this point, light from the lamp is focused to form an image of the filament at the pupil of the eye.

In the microscope, light from the lamp is imaged and reimaged successively by the collector lens in the lamphousing, the condenser, the objective and other optics so that an image of the filament is formed at several conjugate back focal

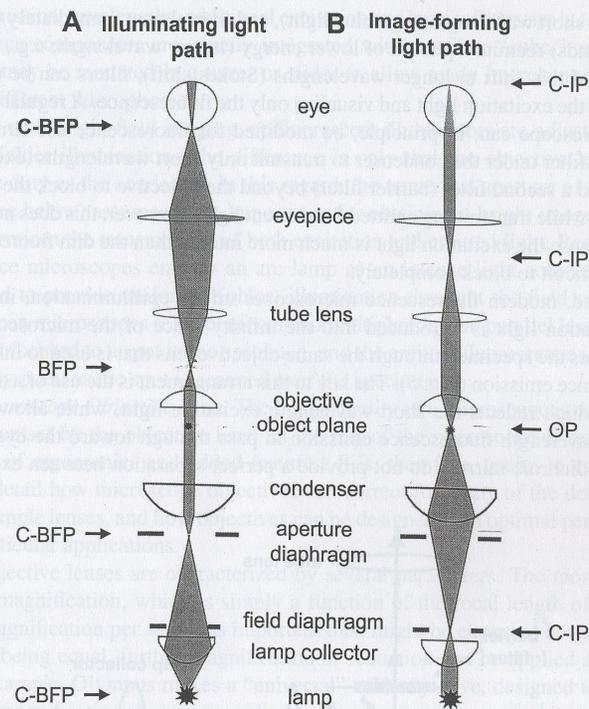


FIG. 4. Diagrams illustrating the paths of illuminating light and image-forming light within an infinity-corrected microscope set up for bright-field Kohler illumination. Note that a point in focus in the object plane (OP) is reimaged at conjugate image planes (C-IP) within the microscope (at the positions of the eyepiece graticule and the field diaphragm in the condenser), as well as at the retina of the eye. Correspondingly, in the illumination light path, the back focal plane of the objective (BFP) is reimaged at conjugate planes (C-BFP) at the exit pupil of the eyepiece and at the aperture diaphragm of the condenser.

planes, corresponding to positions of the aperture diaphragm in the condenser, the back focal plane of the objective, and the eyepoint of the eyepiece. Once again, the angle of light rays at each of these conjugate planes corresponds to the position of an object in the object plane. Conjugate back focal planes are, therefore, good places to put optics such as filters, because any dirt or scratches will not be in focus.

**Fluorescence Microscopy.** Fluorescence techniques form a large part of the biophotonic repertoire. The basic principle of conventional (one-photon) fluorescence is that a molecule (fluorophore) absorbs energy from an incident photon of

relatively short wavelength (e.g., blue light), and then almost immediately (within nanoseconds) reemits a photon of lower energy (longer wavelength; e.g., green). Because of this shift to longer wavelengths (Stokes shift), filters can be used to block out the excitation light and visualize only the fluorescence. A regular bright-field microscope can, in principle, be modified for fluorescence use simply by placing a filter under the condenser to transmit only short wavelengths (excitation filter), and a second filter (barrier filter) beyond the objective to block the excitation light while transmitting emitted fluorescent light. However, this does not work well, because the excitation light is much more intense than the dim fluorescence, and is difficult to block completely.

Instead, modern fluorescence microscopes utilize epiillumination, in which the excitation light is introduced into the infinity space of the microscope and illuminates the specimen through the same objective lens that is used to image the fluorescence emission (Fig. 5). The key to this arrangement is the use of a dichroic mirror, which reflects the short-wavelength excitation light, while allowing the longer wavelength fluorescence emission to pass through toward the eyepieces. Because dichroic mirrors do not provide a perfect separation between excitation

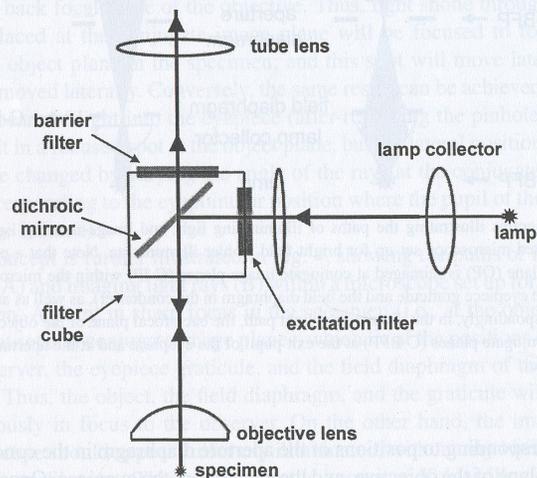


FIG. 5. Diagram of an epifluorescence microscope. A filter cube housing a dichroic mirror, excitation filter, and barrier (emission) filter is mounted in the infinity space behind the objective. Excitation light (usually from an arc lamp) is focused through the excitation filter and reflected by the dichroic mirror so that an image of the arc is formed at the back focal plane of the objective, resulting in even (Kohler) illumination of the specimen. Fluorescent light emitted by the specimen is collected back through the objective, passes through the dichroic, and is imaged by the usual arrangement of tube lens and eyepiece. The barrier filter serves to block any excitation light that was transmitted through the dichroic mirror.

and emission wavelengths, additional excitation and barrier filters are incorporated. All three components are usually integrated within a filter "cube," and several cubes may be mounted in a turret or slider to allow ready interchange for work with different fluorophores.

As with bright-field imaging, epifluorescent illuminators are adjusted to provide Kohler illumination—the difference is only that the objective lens serves double duty as the condenser. In this regard, the numerical aperture of the objective (see below) assumes great importance for obtaining a bright image, because it determines the transmission of both excitation and emitted light. Regular fluorescence microscopes employ an arc lamp as an intense light source, which is focused to provide uniform (Kohler) illumination throughout the field of view. In laser scan microscopes (e.g., confocal and multiphoton) the parallel laser beam is instead focused to a spot in the specimen, and the emitted fluorescence is detected point by point as the spot is scanned over the specimen.

*Microscope Objective Lens.* The image quality of a microscope is determined at the outset by the objective lens: all the rest of the microscope is really just a matter of ergonomics and added features. It is, therefore, useful to consider in more detail how microscope objectives can correct for many of the defects seen with simple lenses, and how objectives can be designed with optimal performance for particular applications.

Objective lenses are characterized by several parameters. The most obvious is the magnification, which is simply a function of the focal length of the lens. The magnification per se is less important than might be expected because, other things being equal, further magnification or reduction can be applied afterward. (For example, Olympus makes a "universal"  $\times 20$  objective, designed to be used with a magnification changer providing final magnifications equivalent to separate objectives with magnifications between  $\times 7$  and  $\times 80$ .) Instead, the numerical aperture (NA) of the objective (defined as shown in Fig. 6A) is the key parameter characterizing an objective.

*NUMERICAL APERTURE.* The numerical aperture is important in two major respects. First, the NA determines the resolving power of the microscope—finer details can be seen with objectives of high NA. An explanation for why this is so was first propounded by Ernst Abbe in the 19th century. In brief, light passing through a specimen emerges such that some remains undeviated (zeroth order), while the remainder is diffracted to form a fan at increasing angles (first order, second order, etc.) from the undeviated light (Fig. 6B). The greater the number of diffracted orders that are captured by the objective, the more accurately the image will represent the original object. However, because of the wave nature of light, a perfect representation can never be achieved. Thus, an infinitely small object appears as an Airy disk: a central spot of finite size surrounded by concentric rings of progressively reducing contrast. The size of this Airy disk decreases linearly with increasing NA of the objective lens and with decreasing wavelength of light (Fig. 6C). Resolution is commonly (although somewhat arbitrarily) expressed in

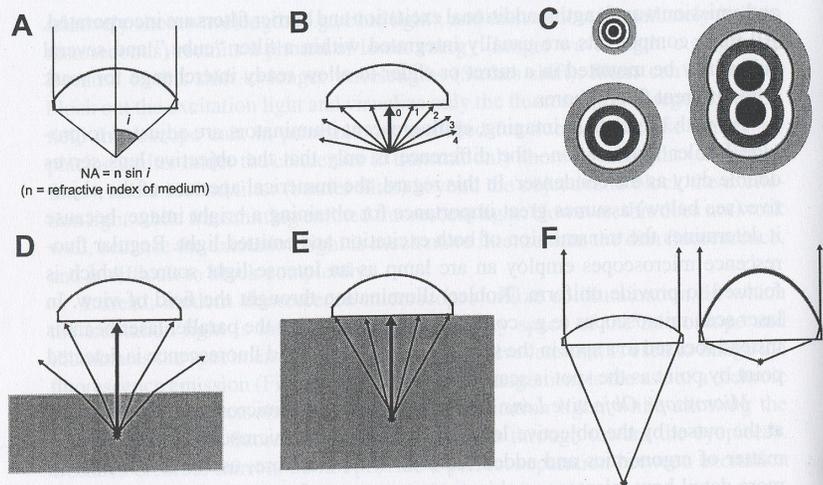


FIG. 6. Numerical aperture and resolution. (A) The numerical aperture (NA) of a lens is given by the sine of the angle  $i$  formed by the most peripheral rays that are collected by the lens, multiplied by the refractive index of the medium (e.g., 1 for a dry objective, 1.3 for a water-immersion objective). (B) Light from a specimen includes both undeviated rays (thick line; zeroth order) and diffracted rays (thin lines; first, second, etc., orders). The greater the number of diffracted orders collected by the objective, the more closely the image will represent the original object. (C) Airy disks and resolution. Objects smaller than the limit of resolution (e.g., 100 nm beads) form images of small, circular diffraction discs (Airy disks). High-numerical aperture lenses (which capture more of the higher orders of diffracted light) produce smaller Airy disks (upper left) than lenses of low numerical aperture (lower left). When two small objects approach close together their Airy disks begin to overlap (right) and, at the limit of resolution, the central spots can no longer be discriminated. (D and E) Higher resolution is achieved by water- or oil-immersion lenses. Illustrated is the imaging of an object in water (shaded) by a dry objective (D) and a water-immersion objective (E). A dry objective fails to capture higher order diffracted light because of refraction at the water-air interface. (F) High-numerical aperture objectives (right) usually have a shorter working distance (distance from the front surface of the lens to the object plane) than do low-aperture objectives (left).

terms of the minimum spacing between two infinitely small objects (conveniently approximated in practice by 50-nm-diameter latex beads or by the fine structure in diatoms) that still allows them to be discriminated (Fig. 6C), leading to the classic Rayleigh equation:

$$D = 1.22/(\lambda/2NA)$$

where  $D$  is the minimum spacing and  $\lambda$  is the wavelength of illuminating light.

Second, the NA determines the light-gathering power of an objective. This is of particular importance for fluorescence microscopy, in which the fluorescence emitted by a specimen is usually dim, but cannot be increased merely by raising the intensity of the excitation light because of problems of photobleaching and phototoxicity to live cells. Each fluorescent molecule emits photons randomly in all directions. It is not possible in practice to collect more than a fraction of these, but the proportion that can be collected through an objective increases in proportion to the square of its NA.

The use of objectives with high NA is, therefore, almost always a good thing; but this does not come without cost. One problem is that the nature of the medium between the specimen and objective sets a fundamental limit to the maximum NA that can be achieved. For example, when an object in water is imaged by a "dry" objective, light rays at increasing angles from the optical axis (containing the higher orders of diffracted light) are refracted outward at the water-air interface, so that they escape the objective (Fig. 6D). Higher apertures can be achieved with an immersion medium (water or oil) between the specimen and objective (Fig. 6E), although this can get messy, particularly with inverted microscopes, with which there is a danger of oil running down into the interior of the lens. (Tricks to avoid this include use of a low-viscosity oil, and placement of a rubber O ring around the barrel of the lens as an oil trap.) Maximum practicable numerical apertures are about 0.9 for dry objectives, 1.2 for water immersion, 1.45 for oil immersion, and 1.65 when utilizing special oil and a coverglass with high refractive index. A second disadvantage is that a high aperture is usually achieved, in part, by minimizing the working distance between the front element of the objective and the object plane (Fig. 6F), which may restrict use with thick specimens and interfere with placement of microelectrodes under the lens. Finally, as noted earlier, problems of spherical and chromatic aberration increase with increasing lens aperture. These can be corrected, but involve more complex designs, so that high-NA lenses are generally more expensive.

**CORRECTION OF LENS ABERRATIONS.** Any reputable objective lens should achieve diffraction-limited performance in the center of the field at a single wavelength, but outside of this limited specification there are several aberrations that may be corrected to different extents. The cost of objectives rises as a steeply nonlinear function of the degree and number of corrections that are applied. These are usually marked on the barrel by abbreviations (which, confusingly, differ slightly between manufacturers). Major corrections may include the following:

**Chromatic aberration:** Achromatic (Achro), fluorite (Fluor), and apochromatic (Apo) lenses, respectively, provide increasing levels of correction for chromatic aberration.

**Field curvature:** All three of the above-described objectives project a curved image. For some purposes this may not matter, but for others (e.g., imaging

cultured cells on a flat coverslip) it does. Plan objectives provide a flat field.

Spectral transmission: Specialized lenses provide enhanced transmission in the UV (e.g., for Fura-2 imaging) or infrared (IR) (e.g., for IR DIC imaging of brain slices).

### Interfacing to Microscopes

Utilizing a regular microscope for biophotonic applications involves getting light into or out of the microscope, often both at the same time. This is facilitated because the manufacturers generally provide several "ports" into the optical path, which, although intended for other purposes, can be readily adapted.

A first consideration is whether a given port provides access to the infinity space of the microscope (e.g., epifluorescence port) or whether it incorporates a tube lens or other focusing optics such that a conjugate image plane is formed (e.g., camera ports). Second, it is best to use a port located close to the objective, because the optical path then includes fewer elements that may absorb or scatter light, and problems of possible misalignment of sliders used to direct light to different ports are obviated. Finally, the use of an inverted microscope is greatly preferable to an upright microscope. Inverted microscopes are equipped with a greater number of ports, which are located near the base of the microscope body at a height convenient for post-mount components fitted to an optical bench.

Several ports may be used at the same time—for example, to introduce UV light for photolyzing caged compounds while simultaneously employing a confocal scanner to monitor signals from visible wavelength dyes. The trick is to arrange so that a dichroic mirror in a port close to the objective will transmit light introduced from more distant ports. For example, a UV-reflecting dichroic placed in the epifluorescence port immediately next to the objective will appear transparent to visible wavelengths passing toward the objective as well as from the specimen into the microscope body.

The epifluorescent port is, in optical terms, the best for providing external optical access to a microscope. It is located in the infinity space, immediately behind the objective, and the availability of sliders or turrets containing several different filter cubes allows the functioning of the port to be readily switched. The use of a dichroic mirror in the port allows, for example, introduction of short-wavelength light for fluorescence excitation while fluorescence emission is viewed through the regular microscope path. Alternatively, a front-surface mirror can be placed in a filter cube so that all light to and from the objective is diverted to the port. A practical disadvantage of the epifluorescence port in inverted microscopes is that it points backward, through the frame of the instrument, so that the infinity space is considerably extended before access is available at the rear of the microscope.

Use of modified side-pointing filter cubes (e.g., the Olympus "laser port") obviates this problem.

The side (video) port of an inverted microscope is perhaps the next best choice. It is located low down on the side of the microscope, and provides convenient access to a conjugate image plane (formed several centimeters away from the microscope body). Light is diverted to the port via a beam splitter cube, which directs all, or a large fraction of, the light to a port. The advantage of a partial split is that the specimen can still be viewed (dimly) through the eyepieces while using the port (e.g., to facilitate alignment procedures), but obviously necessitates some loss of light. Microscopes from some manufacturers incorporate both 100/0% and 85/15% beam splitters, but with other models it is necessary to specify a single type of beam splitter when ordering. Also, some models make provision for a further port, which is accessed from the underside of the microscope body. The 35-mm camera port of an inverted microscope can also be used, but its physical location on the front of the microscope is inconvenient for locating more than small components, and its light path includes intermediate optics to project a magnified real image.

Finally, the phototube of a trinocular eyepiece head again provides access to a conjugate image plane, but its elevated location close to the eyepieces makes interface with external optics inconvenient. However, this may be the only readily available option on an upright microscope, short of machining a custom port that can be sandwiched in the infinity space between the eyepiece head and the microscope body.

### Constructing Optical Systems

Although commercial instruments are now available to serve many biophotonic applications, powerful incentives remain for an investigator to construct his or her own optical system. These include the use of new techniques for which no commercial systems yet exist; the ability to adapt an instrument to suit particular needs; the considerable (typically >5-fold) cost saving that can be achieved; and the intimate familiarity gained of both basic principles and practical implementation. The purpose of the following sections is to demonstrate that such a task is easier than might be expected, and to provide practical methods and tips for the biologist without prior optical experience. This is written on the basis of the author's experience in building confocal and multiphoton imaging and photolysis systems,<sup>8-11</sup> but the general principles are applicable to most biophotonic techniques.

<sup>8</sup> N. Callamaras and I. Parker, *Methods Enzymol.* **307**, 152 (1999).

<sup>9</sup> N. Callamaras and I. Parker, *Methods Enzymol.* **291**, 497 (1998).

<sup>10</sup> N. Callamaras and I. Parker, *Cell Calcium* **26**, 271 (1999).

<sup>11</sup> M. J. Sanderson and I. Parker, *Methods Enzymol.* **360**, [19], 2002 (this volume).

Although optical design is a highly complex business, several factors facilitate the task of a biologist attempting to construct an optical instrument. First, most systems involve interfacing external optics to a microscope, where the hard work in terms of optical design (corrections for spherical and chromatic aberrations, flatness of field, etc.) has already been incorporated into the objective lens (which is why lenses can cost many thousands of dollars). What emerges from the microscope port are parallel beams of light that can be imaged without introducing appreciable distortions by simple lenses of low numerical aperture, and without knowledge of more than the elementary lens formulas. Second, a large industry has developed to support the laser and optical telecommunications sectors, so that a vast range of optical (lenses, prisms, mirrors, etc.) and optomechanical (tables, posts, mirror mounts, etc.) components are available "off-the-shelf." Finally, many applications involve the use of lasers, which serve as an almost perfect light source, emitting a narrow, parallel beam of (usually) monochromatic light. Laser beams can be directed at will by steering mirrors, allowing the layout of optical components to be arranged for physical convenience, and can be focused by simple lenses without problems of spherical or chromatic aberrations. Assembling an optical system, therefore, usually involves little more than bolting together standardized parts (a grown-up Lego set), and can be accomplished with only a fairly rudimentary background in optics.

The following sections deal with (1) photonic components for generating, manipulating and detecting light (lasers, lenses, photomultipliers, etc.), (2) optomechanical components (mounts, tables, etc.) for assembling the optical components together in the correct alignment, and (3) an example illustrating the design and construction of a simple biophotonic instrument.

### Photonic Components

#### Light Sources

**CONVENTIONAL SOURCES.** Commonly used nonlaser light sources include tungsten-halogen bulbs and arc lamps. In all of these, light is emitted in all directions from a source of finite size (e.g., a filament array covering a few square millimeters in a halogen bulb, or a <1-mm sphere in a small arc lamp). Unlike with a laser (which behaves as a perfect point source), it is impossible to effectively collect all of the emitted light or to focus the source down to a diffraction-limited spot. Furthermore, light from conventional sources is polychromatic (spanning a wide range of wavelengths), and is incoherent (light waves are not in phase). Nevertheless, conventional sources are generally preferable to lasers for applications requiring uniform illumination of extended areas rather than the formation of an intense, focused spot.

Halogen lamps (Fig. 7F) emit a smooth, continuous spectrum, with most energy in the infrared and progressively less at shorter wavelengths. They provide little

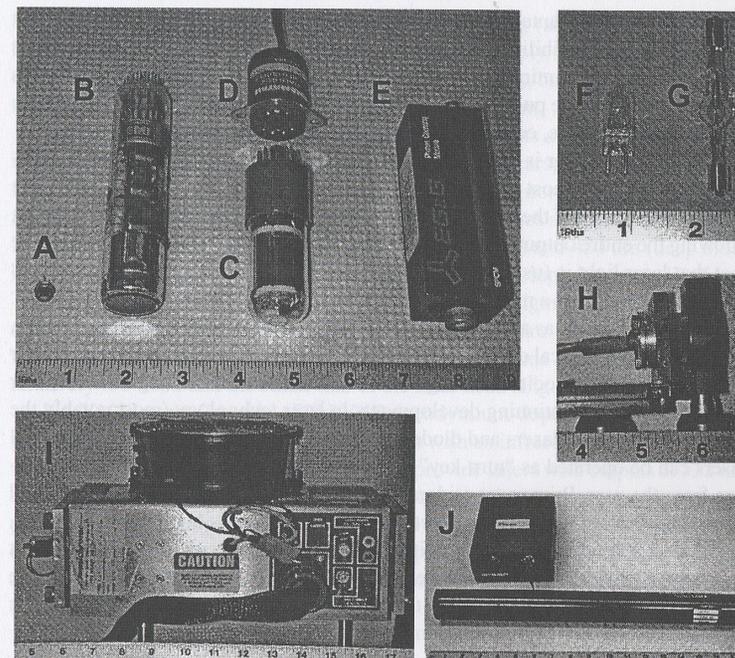


FIG. 7. Detectors (A-E) and light sources (F-J). Scales are in inches. (A) A PIN photodiode. (B and C) Head-on and side-on photomultiplier tubes. (D) Photomultiplier socket with built-in high-voltage power supply. (E) Avalanche photodiode photon-counting module. (F) Tungsten-halogen light bulb. (G) Xenon arc lamp bulb. (H) Laser diode with collimating lens. (I) Air-cooled argon ion laser. (J) Helium-neon laser and power supply.

useful UV output. Advantages include simplicity, low cost, long life, and the ability to obtain a highly stable output when operated from a well-regulated DC power supply.

Arc lamps work by establishing an electrical discharge in a pressurized gas enclosed in a quartz bulb (Fig. 7G). The arc is smaller and more intense than the filament of a halogen lamp, so that light can be collected more efficiently and focused much more intensely on a small specimen. Also, arc lamps have a strong output in the UV (down to <250 nm), and provide a more "white" illumination in the visible. Xenon arc lamps show a relatively uniform spectrum throughout the UV and visible spectra, whereas the output of mercury arc lamps is concentrated in a few sharp peaks (365, 46, 546, and 577 nm). Disadvantages shared by all arc lamps include a less stable output (the arc position tends to wander, and bulbs

should always be mounted vertically to minimize this); the short life and high cost of bulbs; and the possibility that bulbs will explode, destroying not only themselves but also the lens and mirror within the lamp-housing! Furthermore, xenon lamps require a high-voltage pulse to "ignite" them, which can damage nearby electronic equipment (amplifiers, computers) if turned on before the lamp.

**LASERS.** The laser is the key invention that has made many biophotonic techniques possible. In most instances (e.g., confocal microscopy, laser trapping) the important property of the laser beam is that light rays are almost perfectly parallel, allowing the entire output to be focused to an intense, diffraction-limited spot. The fact that laser light is (usually) monochromatic is an incidental advantage; its coherence is sometimes a nuisance, leading to interference effects and laser speckle. Whereas lasers serve as an almost perfect light source from an optical perspective, they may have practical drawbacks including high cost, short lifetime, high power consumption and associated heat generation, and need for cooling fans or water supply. However, continuing developments in laser technology (most notably the availability of diode lasers and diode-pumped systems) now mean that almost all lasers can be operated as "turn-key" devices without requiring any special expertise from the user. Primary considerations in selecting a laser include the desired operating wavelength, required power, whether the beam is continuous or pulsed, and the beam quality. The best quality is known as TEM<sub>00</sub>, meaning that the light intensity across the width of the beam is distributed as a Gaussian profile. The following list describes some of the types of laser commonly used for biophotonic applications, but is by no means comprehensive.

**Argon ion lasers:** The argon ion laser has been the workhorse for laser-scan fluorescence imaging for many years. Argon ion lasers produce a bright blue line at 488 nm, which is well matched to many fluorophores [e.g., fluorescein isothiocyanate (FITC), green fluorescent protein (GFP) variants, and numerous green Ca<sup>2+</sup> indicators]. Small air-cooled units (Fig. 7I) are available with powers up to 100 mW, which is more than sufficient for fluorescence imaging. Multiline argon ion lasers produce outputs also at 457 and 514 nm, which can be selected by interference filters. Argon-krypton lasers further add lines at 647 and 676 nm; but they have a poor reputation for reliability. A drawback of ion lasers is their remarkable inefficiency (1 kW of electricity gives <100 mW of light), resulting in the need for a cooling fan. This is best located remotely (the space above the ceiling tiles is a good place) to reduce noise and heating, and coupled to the laser through flexible tubing. The laser tube has a finite lifetime (about 3 years under typical operating conditions), which shortens steeply as the laser is operated at higher powers. A solid-state 488-nm laser has been introduced (Sapphire; Coherent, Auburn, CA) as an argon ion laser replacement, and offers much higher efficiency (no cooling required) and a small laser head.

**Helium-neon lasers:** Helium-neon lasers are simple and inexpensive, with models available giving lines at 543 nm (green), 594 nm (yellow), 612 nm (orange),

and 633 nm (red). They are usually packaged as a long (about 1-m) cylindrical laser head, and a small, separate high-voltage power supply (Fig. 7J). Their power output is only a few milliwatts at the shorter wavelengths, but can be a few tens of milliwatts for red He-Ne lasers. Beam quality is excellent.

**Laser diodes:** Semiconductor lasers producing outputs at several wavelengths in the red and infrared have been available for several years, with powers ranging from <1 mW to >1 W. These long wavelengths are not generally useful for fluorescence applications, but are applicable for techniques such as laser trapping. However, novel diode lasers operating at 400 nm (violet) and 450 nm (deep blue) have become available, and are likely to find numerous biophotonic applications. The light output from all laser diodes is diverging and astigmatic, so special optics (often packaged together with the diode in a module) are needed to generate a well-collimated beam. Advantages of diode lasers include their high efficiency, relatively low cost, and the small size of the laser head (Fig. 7H).

**Nd:YAG lasers:** Crystals of Nd:YAG (neodymium-doped yttrium aluminum garnet) form a highly efficient lasing medium with an infrared output at 1064 nm, which can be frequency doubled or tripled to provide lasers with outputs at 532 nm (green) and 355 nm (UV). Models using different mechanisms to pump the crystal provide either a continuous beam or a pulsed output (e.g., pulses of about 5 ns in duration at repetition rates up to 20 Hz). The power during each pulse can be high, and can be utilized, for example, for cell ablation or localized photolysis of caged compounds.

**LASER SAFETY.** Laser systems should be constructed with covers that entirely enclose the beam during normal operation. Use a low optical table so that the laser beam is below eye level, even when sitting. Post warning signs, and restrict access to the room. If a laser beam is exposed, wear appropriate protective eyewear unless you need to see the beam in order to align it. In that case, attenuate the laser power as much as possible. Beams that you cannot see (UV and IR) are more dangerous than those you can, and pulsed lasers (e.g., Nd:YAG) cause greater damage than continuous lasers of equivalent power. Accidents happen most often when the user is tired or frustrated. Years of experience in working with lasers does not confer immunity to accidents; rather, it induces complacency. Finally, although the main danger from the relatively low-power laser beams used in biophotonic techniques is limited to eye damage, attempts to repair high-voltage power supplies (e.g., in argon ion and flash lamp-pumped lasers) can result in fatal electrocution.

The back of a white business card is the standard tool for visualizing laser beams. If the beam is in the UV, it can be seen by rubbing the card with a yellow fluorescent highlighter pen. Infrared detector cards allow visualization of IR beams—but these do not work well and IR viewers are a better solution.

**Detectors.** Light detectors can work in one of two modes: analog or photon counting. In analog mode the electrical output varies in a continuously graded fashion with the photon flux incident on the detector. In photon-counting mode,

a discrete electrical pulse is generated for each photon that is detected. Photon counting is not inherently more sensitive than analog detection, but provides lower noise (analog detection introduces "multiplicative" noise, as the electrical signals produced by successive photons of the same energy vary in amplitude), and is preferable for low light levels. Other important characteristics to consider when choosing a detector include quantum efficiency (the percentage of incident photons that actually generate a signal), dark current or noise (the analog current or false counts generated in complete darkness), relative sensitivity at different wavelengths, and speed of response. Available, highly sensitive detectors are based on both vacuum tube technology (photomultipliers; Fig. 7B and C) and solid state technology (photodiodes; Fig. 7A and E).

**PHOTOMULTIPLIERS.** A photon incident on the cathode of a photomultiplier dislodges a photoelectron, which is then amplified by acceleration through a cascade of electrodes (dynodes) held at increasing high voltages. The result is that individual photons produce current pulses at the anode that are sufficiently large that essentially no further noise is introduced by subsequent amplification or electrical processing. The photomultiplier output can be used in photon-counting mode by employing electrical circuitry that discriminates signals above a certain threshold, and converts them to stereotyped pulses of fixed amplitude and duration. Alternatively, the anode current can be simply filtered and converted to an analog voltage signal by using a current-to-voltage converter. Advantages of photomultipliers include their large detector area (25 mm in diameter or greater), low dark count ( $<10$  counts  $s^{-1}$  on selected tubes; even lower if cooled), wide dynamic range, and ability to cope with high photon fluxes ( $>10^8$  counts  $s^{-1}$ ). Drawbacks include a relatively poor quantum efficiency (typically  $<20\%$  and decreasing at longer wavelengths), large physical size, and susceptibility to magnetic fields. Photomultipliers require a high-voltage supply, a resistor chain to provide dynode voltages, and a sensitive current amplifier. However, these are available as modules from manufacturers, and can even be obtained built into the photomultiplier socket (Fig. 7D), so the use of photomultipliers is simpler than might be anticipated. The photomultiplier gain can be readily controlled simply by varying the high-voltage supply; this is a commonly used approach, but not theoretically ideal because an optimal signal-to-noise ratio is achieved only throughout a narrow voltage range.

**PHOTODIODES.** Light incident on a semiconductor junction generates an electrical current. In regular (PIN) photodiodes (Fig. 7A) each photon produces only a single electron charge, which becomes swamped by noise originating in the diode itself and in the subsequent amplifier. Such photodiodes are, therefore, not suitable for photon counting or for low light level detection. However, they have a high quantum efficiency (60% at 550 nm, increasing toward the infrared), are small and inexpensive, and respond rapidly. Although wide-area photodiodes are available, their noise decreases and speed of response increases as the detector area is decreased, so photodiodes are best suited to applications in which light of moderate or high intensity can be focused down to a small spot.

Avalanche photodiodes combine many of the advantages of photomultipliers and solid state detectors. They work by applying a high reverse bias (voltage) across a specialized photodiode. An incident photon causes a transient breakdown, leading to a large flood of electrons so that the diode provides inherent gain (many electrons from a single photon) similar to a photomultiplier. Avalanche diodes provide the high quantum efficiency of a photodiode together with a low dark count similar to a photomultiplier. They are generally used in photon-counting mode, and offer unmatched sensitivity at low light levels. Complete modules (Fig. 7E) are available that include the diode and associated circuitry: only a low-voltage power supply is required, and the output gives a TTL pulse for each detected photon. Disadvantages of avalanche photodiodes include their small detector area (a few hundred microns for lowest dark noise), and relatively low maximum count rate (about  $10^7 s^{-1}$ ).

### *Optical Components*

**Mirrors.** Use of front-surface mirrors is an absolute requirement to avoid secondary reflections from the front glass surface of a back-coated mirror. However, despite the use of protective coatings, the front coating is delicate; do not touch it with your fingers, and try to minimize the need for cleaning. Silver-coated mirrors are a good general purpose solution, and have reflectivity better than 95% from about 450 nm well into the infrared. For use at shorter wavelengths, UV-enhanced aluminum mirrors reflect down to  $<200$  nm, but their reflectivity is only about 85%. Dielectric mirrors provide the ultimate in reflectivity ( $>99\%$ ) when every photon counts, but are more expensive than metal mirrors, and work only over specified wavelength bands (i.e., separate mirrors are needed for UV, visible, and IR), which depend somewhat on the angle of incidence and polarization of the light.

**Spectral Filters.** Two types of filters are available that selectively transmit particular wavelengths of light: colored glass filters, (the absorption of which varies with wavelength), and interference filters made by combining many thin layers of dielectric materials to produce constructive and destructive interference in transmitted light. Factors to consider when choosing a filter include the desired wavelength range for transmission, how steeply transmission cuts off outside this range, the peak transmission, and how well the filter blocks wavelengths well outside its pass band.

Colored glass filters work superbly well as long-pass filters. Their transmission at long wavelengths is high ( $>99\%$ ), blocking at short wavelengths is excellent ( $<10^{-5}$ ), and the cut-on (increase in transmission with wavelength) is sharp. They are simple, inexpensive, and available for a wide range of wavelengths and in several sizes. For short- and band-pass applications, however, colored glass filters serve less well. For these applications, interference filters are generally preferable. They allow a precise control center wavelength, bandwidth, and steepness of cut-on and cut-off, and are available with characteristics tailored to match the spectra of many fluorophors. One characteristic to note is that, unlike colored glass filters,

interference filters are sensitive to the angle of the incident light, and are thus best used with fairly well-collimated (parallel) light beams. In some instances this characteristic can be utilized for "tilt tuning": shifting the transmission to shorter wavelengths by deliberately tilting the filter in the light path. A further characteristic is that they may display unexpected behavior outside their specified spectral range—for example, a filter designed to transmit at 400 nm may also transmit at 800 nm. To mitigate this latter problem, interference filters are often cemented together with a colored glass filter. Such composite filters should be mounted with the "shiny" side facing a strong light source.

Dichroic mirrors are a form of interference filter specifically designed to reflect a range of wavelengths while transmitting the remainder. They are mostly used to reflect short wavelengths and transmit longer wavelengths, but dichroic mirrors with band-pass and long-wavelength reflection are also possible. Like interference filters, the characteristics of dichroic mirrors depend on angle, and they are usually specified for incidence at  $45^\circ$ . They should be mounted with the correct orientation (reflective surface facing the incident light) to minimize spurious reflections from the back surface of the glass substrate.

**Neutral Density Filters.** Neutral density (ND) filters made with partially reflecting metal coatings on a glass substrate provide a convenient means of attenuating laser and other light beams with fairly uniform attenuation over a wide (400- to 2000-nm) spectral range. They are usually specified in terms of optical density [OD =  $-\log(\text{transmission})$ ; e.g., an OD of 1 will transmit 10% of the incident light, an OD of 2 transmits 1%, etc.]. Filters should be inclined at a small angle to a laser beam, as light reflected back into the laser cavity can cause unstable fluctuations in power output. ND filters are available as individual components (Fig. 8D); but these tend to become covered by fingerprints and are easy to lose. A better solution is to use ready-assembled wheels containing sets of ND filters (Fig. 8F). For example, a unit with two six-place wheels containing filters graduated in steps of 0.1 and 0.5 OD allows selection of a wide range of densities between 0 and 3.0 (100 to 0.1% transmission) in increments of 0.1. Alternatively, continuously varying ND filters are available, constructed both as glass disks, with density varying with rotational angle (Fig. 8E), and as rectangular filters, with density varying linearly with length. Such continuous filters are most useful when a smooth control of intensity is needed, without requiring precise knowledge of the degree of attenuation.

**Lenses.** Simple (singlet) lenses are available with both surfaces curved (e.g., equiconvex) or with one flat surface (e.g., planoconvex). The latter is preferable for focusing parallel and near-parallel beams, and should be oriented so that the "work" done in bending the rays is shared between both surfaces (i.e., with the flat surface adjacent to the focal point). Equiconvex lenses are preferred for imaging at magnifications close to unity ( $< \times 5$ ), as their symmetry causes aberrations to cancel. Meniscus lenses (with surfaces of opposite curvature) are used to change the focal length of another lens.

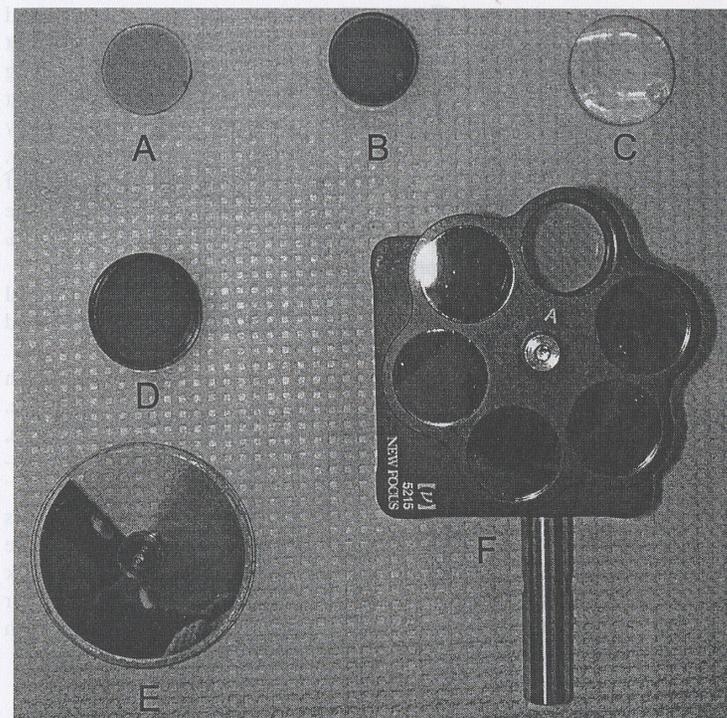


FIG. 8. Optical components. (A) Neutral density filter. (B) Interference filter. (C) Convex lens. (D) Polarizing filter. (E) Circular continuously variable neutral density filter. (F) Six-position neutral density filter wheel.

Aspheric lenses can be made with short focal lengths while minimizing spherical aberration, and are good for high throughput applications, such as concentrating light onto the sensitive area of a detector or collecting the maximum possible light from a lamp. However, their optical quality is generally poor, and aspheric lenses are not well suited to form sharp images.

Cylindrical lenses focus light in only one dimension, and can thus be used to focus a laser beam as a line, rather than a spot.

Achromatic doublet lenses comprise two closely spaced, often cemented, combinations of converging and diverging elements with differing refractive index. Their focal length remains virtually constant across the visible spectrum (i.e., almost no chromatic aberration), and spherical aberration is also much reduced, so that they are superior to singlet lenses even for monochromatic applications.

*Types of Glass and Optical Coatings.* The extent to which light is transmitted through an optical component (lens, prism, etc.) depends both on how much light is reflected at each air–glass interface, and on how much is absorbed in passing through the glass. In typical use, reflection from an untreated glass surface is about 4%, amounting to an 8% loss in light passing through a single lens. This rapidly compounds in multielement systems and, in addition to the reduction in intensity, multiple reflections increase stray light and reduce image contrast. The use of antireflective coatings substantially mitigates this problem, and most optics can be ordered with broad-band antireflective coatings that reduce reflection to <0.5% per surface over a specified wavelength range (e.g., 375–650 nm).

Absorption of light by the glass substrate is usually not an issue. Regular optical glass (BK7) transmits well at wavelengths from about 350 to 2000 nm, and fused silica can be used for wavelengths deeper into the UV (down to 200 nm).

*Cleaning Optics.* If an optic needs cleaning, first blow off the dust, which otherwise acts like sandpaper. Then clean it gently with a solvent and lens tissue. Always use lens tissue (not a Kimwipe or clothing), and never use dry tissue, which will scratch. A good solvent is a mix of 60% acetone and 40% methanol—both of high purity. Gentle cleaning is best accomplished by the “drop and drag” technique. Place the dusted optic face up on a clean surface. Lay an unfolded lens tissue on top of it, apply a few drops of solvent, and slowly drag the soaked tissue across the face of the optic. For heavier cleaning, fold a tissue several times, soak it with solvent, and slowly wipe the folded edge across the optic, using fingers or plastic forceps. Do not touch the part of the tissue used for cleaning, and use each tissue only once.

#### *Optomechanical Hardware*

The method of construction described and recommended here is based on laying out optical components, using standard mounts affixed to an optical breadboard or table (Fig. 9). This approach offers great flexibility. Off-the-shelf parts are available to cover most eventualities, and components can be readily adjusted, added, or recycled to build new projects. A basic stock of parts is a good lifetime investment, as they will never wear out and are unlikely to become obsolete.

*Breadboards and Tables.* Optical systems are most conveniently built on some form of optical “breadboard”: a flat, rigid metal surface, usually drilled and tapped with a regular grid of mounting holes on 1-inch (or 25-mm) centers, and used to support components on pillar or postmounts (see below). These are available in a wide range of sizes (from a few inches to room-filling) from most optical suppliers. For small instruments (up to about 2 ft<sup>2</sup>) a thick sheet of aluminum suffices. However, for larger sizes a thicker construction is used, comprising a honeycomb core (providing high rigidity for low weight) sandwiched between upper and lower

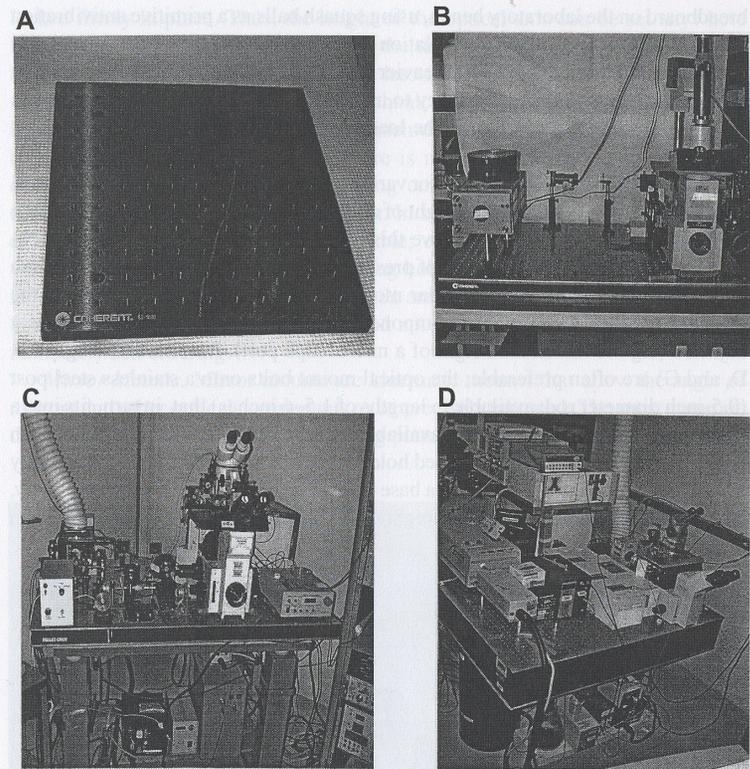


FIG. 9. Optical base plates, breadboards, and tables. (A) Small (1-ft<sup>2</sup>) base plate, made from aluminum plate with tapped holes at 1-inch centers. (B) Optical breadboard, used to construct a total internal reflection microscope. Little vibration isolation was required, so the breadboard is simply laid on the laboratory bench. (C) Larger breadboard used as a free-standing table to construct a confocal microscope. (D) Large (8 × 3 ft) optical table with air-suspended feet, used to construct a multiphoton microscope.

steel plates. These are available as relatively thin and light breadboards that can be laid on top of a regular laboratory bench, and as thicker, heavier units that function as independent tabletops. Optical tables with sizes up to about 4 × 3 ft can be put into place without assistance, but larger sizes require professional installation.

The method used to support the breadboard or table depends on the degree of vibration isolation that is needed. In a rigid building without any major vibration source (e.g., nearby traffic, elevator machinery) it may suffice simply to place a

breadboard on the laboratory bench, using squash balls as a primitive antivibration mount. The ultimate vibration isolation is achieved by self-leveling pneumatic isolators, which work best with heavier tables. However, these are not without their problems, including a tendency to instability if the load on the table changes (a carelessly rested elbow), or if the load is asymmetrically distributed or has a high center of gravity.

**Posts and Pillars.** The holders for various optical components (mirrors, lenses, etc.) are typically mounted at a height of a few inches above the optical table. Two main systems can be used to achieve this. The first employs steel pillars: 1-inch-diameter rods, available in a range of preset lengths, which are clamped down with metal forks (Fig. 10A and B). Pillar mounts provide high rigidity, but have the disadvantage that the height of components cannot be readily and continuously adjusted (e.g., to match the height of a microscope port). Post mounts (Fig. 10C, D, and G) are often preferable; the optical mount bolts onto a stainless steel post (0.5-inch diameter rod, available in lengths of 1.5–6 inches) that, in turn, fits into a post holder (1-inch-diameter tube, available in various heights). The post holder can either be screwed directly to a tapped hole in the breadboard or, to allow flexibility in lateral positioning, mounted via a base plate (Fig. 10E and F). Most importantly, a spring-loaded clamp allows the height of the post to be easily adjusted, and then

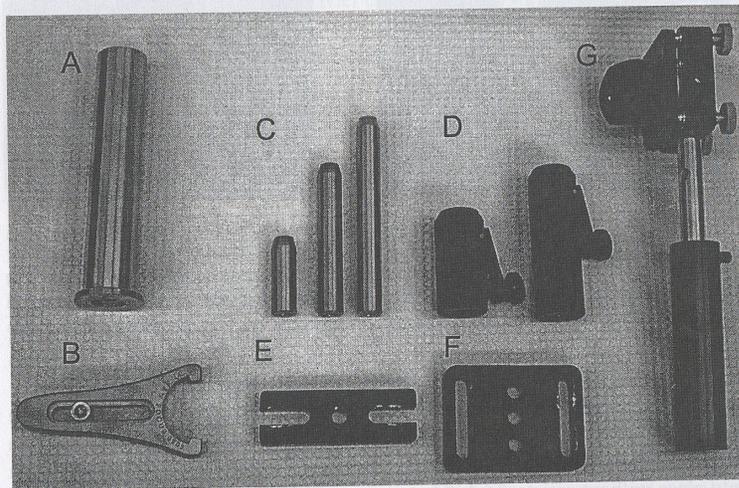


FIG. 10. Pillar and post mount components. (A) Pillar mount. (B) Clamping fork for the pillar. (C) Mounting posts of varying length. (D) Post holders of varying length. Note the spring-loaded clamping devices (lever and screw on right) which secure the post. (E and F) Narrow and wide base plates. A post holder is bolted onto the central hole, and the assembly is then attached to an optical table using the slotted holes. (G) Assembled unit, with a mirror mount attached to a post and post holder.

locked firmly in place. Thus, the height of the optical axis can be set anywhere between about 2.5 and 10 inches above the table top—although it is better kept fairly low if possible, as longer posts are less stable and tend to vibrate.

**Imperial and Metric Sizing.** Optomechanical components are available in both imperial (e.g., 1-inch hole spacing, 0.5-inch-diameter posts) and metric (25-mm hole spacing, 12-mm posts) sizes. There is no functional reason to prefer one over the other, and when first purchasing components it is simply a matter of deciding on one system and sticking to it. In some cases the sizes are sufficiently close that components are interchangeable; e.g., 1-inch (25.4-mm) base plates will work on tables with 25-mm mounting hole arrays. However, there are many incompatibilities (mounting screw threads are different, and inch posts will not fit metric post holders), so care is needed not to mix up metric and inch catalog numbers when ordering.

**Mirror Mounts.** Mirror mounts are kinematic mounts that allow the angle of mirrors and other optical components to be precisely adjusted. The most common design (Fig. 11A) comprises a mounting plate supported at three points, two of

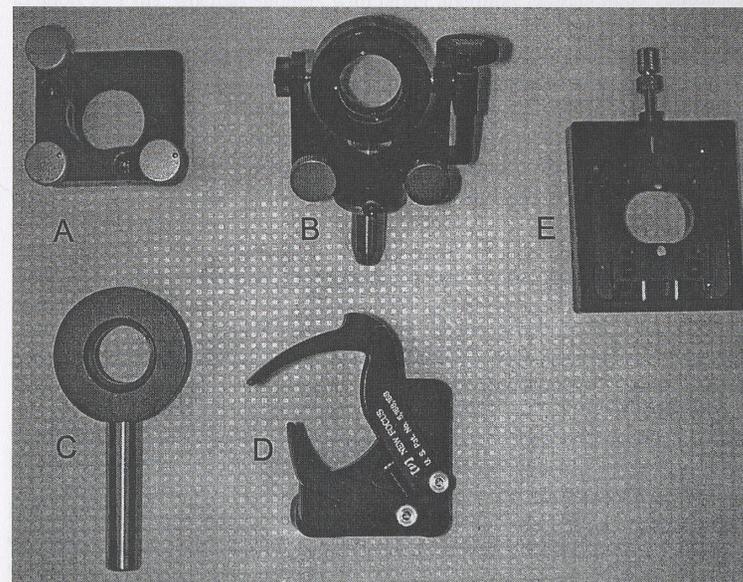


FIG. 11. Optical component holders. (A) Standard mirror mount. (B) Gimbal mirror mount. (C) Mount for a 1-inch-diameter lens or filter. (D) Adjustable, self-centering mount (Opticlax; New Focus, San Jose, CA), capable of holding components of varying diameters up to 2 inches. (E) Translation stage, providing precise lateral positioning.

which have adjusting screws that independently control vertical and horizontal tilt. The expense of top-quality units is well justified, as these employ fine-pitch screw threads and sapphire seats that promote smooth positioning and long-term stability. Versions with the adjusting screws on top (rather than on the back) allow alignment while a laser beam remains fully enclosed beneath covers, but have more backlash than regular models and are thus best avoided unless they offer a particular ergonomic advantage. Regular mirror mounts introduce a slight translation of the laser beam in addition to rotation (because the mounting plate moves forward as it is tilted). This is not usually a problem, but if it is, gimbal mounts (Fig. 11B) allow pure rotation around an axis in the center of the front plane of the mirror.

**Component Holders.** Simple component holders (e.g., Fig. 11C) allow the mounting of optics such as lenses and filters with standard diameters (e.g., 1 inch/25 mm). For optics of nonstandard size, the Opticlaw mount (New Focus, San Jose, CA; Fig. 11D) provides an ingenious solution. Other available holders are threaded to accept microscope objectives, and specialized rotary holders can be used with polarizers and prisms. Linear translation slides (Fig. 11E) allow for fine lateral movement of components, and are available with manual and motorized actuators.

In most instances optics can be held in place by the set-screw or threaded ring supplied with a holder, but improvisation is sometimes needed. Double-sided adhesive tape works well for components with flat surfaces (e.g., prisms), and Blu-tack (Bostik, Leicester, UK) is a semipermanent, but easily removable, material used to fix lenses and other small components. UV-curing adhesive is the method of choice to cement optical components together. Quick-setting epoxy is good for permanent mounting of optical and mechanical components, but cyanoacrylate adhesive ("Super Glue") is best avoided because its vapor deposits a film on optics.

**Scanners.** As described above, it is possible to scan a focused spot of laser light across a specimen by varying the angle of a laser beam introduced into a microscope at a conjugate back focal plane. This is most conveniently done with a front-surface mirror that is rotated by a galvanometer. Complete units are available from two major manufacturers [Cambridge Instruments (Cambridge, MA) and General Scanning/Lumonics (Bedford, MA)] and comprise a motor (moving coil or moving magnet) with an integral rotational position sensor, an attached mirror, and a separate driver/servo printed circuit card. Use of a negative feedback servo ensures that a given input voltage results in a precise angle of rotation of the mirror. The speed of response depends on the inertia of the rotating mass, so that higher speeds can be attained with smaller mirrors. Current systems with mirrors of about 3 mm (sufficiently large for most purposes) respond to small step changes within 200  $\mu$ s, and can be used for linear (sawtooth waveform) scanning at repetition rates of 1 kHz or higher. However, scanning at video rate requires even faster rates (15 kHz), which cannot be attained by linear galvanometer systems. One solution

is to use a resonant mirror, which is designed to rotate sinusoidally at a fixed frequency determined by its mechanical construction (a rotational equivalent of a tuning fork). Another approach (although one that appears to have seen little practical implementation) is to use a multifaceted polygonal mirror rotating at high speed.

Scanning in two dimensions ( $x$ - $y$ ) presents a problem in that two mirrors, rotating around orthogonal axes, are generally required, and both cannot be placed together at a conjugate back focal plane. Various solutions are possible. The simplest is to locate both mirrors as close to each other as possible, with the conjugate back focal plane located midway between them so as to minimize scanning errors. A second is to use relay optics to reimagine the conjugate plane from one mirror to the second. A third is to adopt a single-mirror system, in which a small galvanometer providing the fast  $x$  scan is mounted on a larger galvanometer so that rotation of the entire  $x$ -galvanometer/mirror assembly provides the slower  $y$  scan.

**Shutters.** Uniblitz shutters (Vincent Associates, Rochester, NY) are simple, robust electromechanical devices, available with apertures from 2 to 35 mm. Units with small apertures are suitable for switching laser beams and allow open times as short as 1 ms, whereas shutters with larger apertures operate more slowly but can be used in the illumination or imaging light paths of a microscope. Special driver units are needed to operate the shutter, and can be either "dumb" (with the open time determined by the duration of a TTL pulse) or include built-in timers. Shutter housings carry a threaded hole for mounting on standard optical posts, and can be ordered with adaptors to fit microscopes from all major manufacturers. Shutter blades that face powerful arc lamp or laser sources should be uncoated (shiny steel), but black coatings are available to reduce reflections for low light level applications.

Pockels cells and acousto-optic devices provide means for shuttering and modulating laser beams at rates (microseconds) much faster than is possible with mechanical shutters.

#### *Example: Building a Total Internal Reflection Microscope*

This section gives a practical example of constructing a relatively simple biophotonic instrument—a microscope for evanescent wave imaging—to illustrate the processes involved in going from theory to practical implementation.

**Theory.** When light undergoes total internal reflection at an interface from high to low refractive index (Fig. 1E) it travels a small distance into a medium of low refractive index. This evanescent wave can thus be used to excite fluorescence that is confined to a thin plane immediately next to the interface, thereby providing an optical sectioning effect.<sup>12</sup> The great advantage is that the section is

<sup>12</sup> D. Axelrod, *Methods Enzymol.* **361**, in press (2003).

much thinner (about 30–100 nm) than can be achieved by confocal microscopy. A major disadvantage is that only objects immediately adjacent to the interface can be viewed and, unlike confocal microscopy, the section cannot be focused throughout a specimen. Total internal reflection microscopy (TIRFM) is readily applicable to live cells, because the refractive indices of water and biological tissue are appreciably lower than that of glass.

The simplest way to implement TIRFM is to place a fluorescent specimen on a glass block, and introduce excitation light through the side at an appropriate angle to provide total internal reflection. However, the specimen must then be viewed from above, which both limits physical access and degrades optical quality, because light must pass through the thickness of the specimen before reaching the microscope objective. A more elegant approach is to utilize excitation by an off-axis beam introduced through the objective lens of an inverted microscope. This is possible using oil-immersion objectives with high NA, so that the light rays from the periphery of the lens make an angle sufficiently shallow to undergo total internal reflection at the interface between a cover glass and aqueous medium. Fluorescence excited within the evanescent layer can then be viewed through the objective (as with conventional epifluorescence microscopy; Fig. 5) without passing through intervening tissue, and the space above the specimen is open for manipulation (positioning of pipettes, addition of drugs, etc.).

**Optical Design.** For through-the-lens TIRFM laser light must be introduced at the periphery of a high-aperture objective lens so as to produce Kohler illumination of the evanescent wave at the specimen. Figure 12A shows an optical layout to achieve this result, with the light path of illuminating rays shown shaded. A parallel laser beam is focused by lens L1, and reflected by a dichroic mirror in the microscope epifluorescence port to form a spot at the back of the objective lens. The beam is directed onto the dichroic by mirror M3, which is mounted on a linear translation stage so that the position of the spot can be moved across the back focal plane and thereby change the angle of incidence of light emerging from the objective to achieve optimal conditions for TIRFM. Fluorescence emitted from the specimen is collected by the objective and imaged through the microscope eyepieces after passing through the dichroic mirror and a long-pass barrier filter to block laser light. Lens L1 acts as a tube lens, so that a conjugate image plane is formed behind the lens. An aperture placed at this point thus appears in sharp focus through the eyepieces, and can be used to restrict the illuminated area in the specimen. The aperture needs to be uniformly filled by laser light to give even illumination, but the beam emerging from the laser is too narrow to provide a sufficient field. Thus, a beam expander is used to fill the aperture and provide a usefully large illuminated area within the microscope field of view.

**Practical Implementation.** The key component in implementing this design is a newly available lens from Olympus (Plan Apo  $\times 60$ , oil immersion, TIRFM)

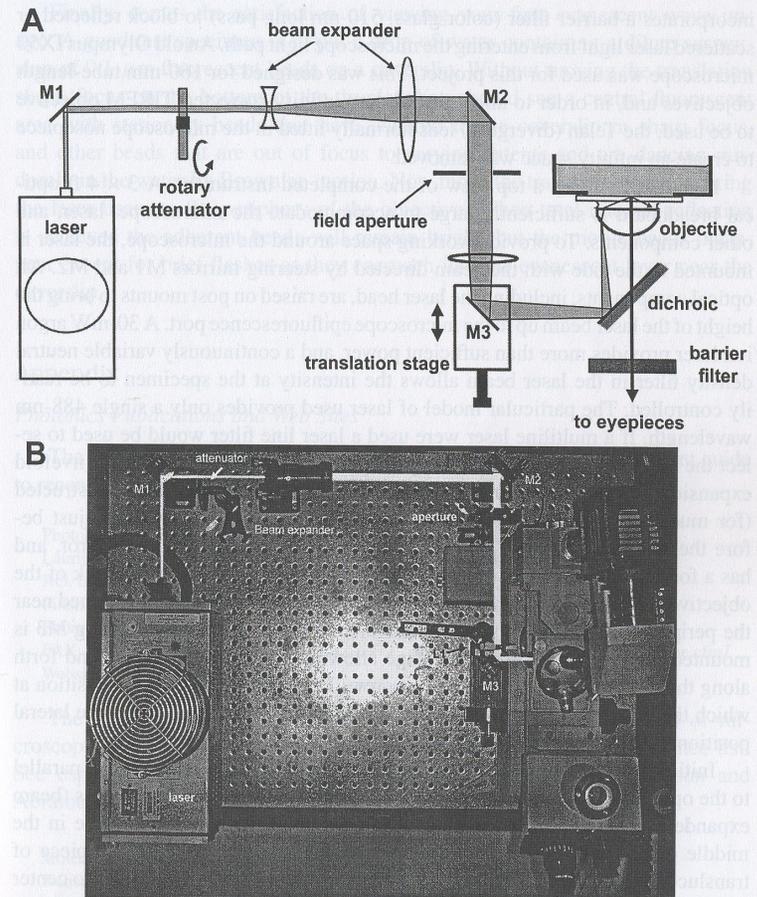


FIG. 12. Schematic layout (A) and final design (B) of a through-the-lens total internal reflection microscope. See text for further details.

that provides an extremely high numerical aperture (1.45) while still working with cover glasses and immersion oil of regular refractive index. This is mounted on an inverted microscope, and a dichroic mirror reflecting  $\lambda < 500$  nm is fitted in a custom-modified cube mounted in the epifluorescence port so that laser light can be introduced from the side, rather than the rear of the microscope. The cube also

incorporates a barrier filter (color glass, 510-nm long pass) to block reflected or scattered laser light from entering the microscope light path. An old Olympus IX50 microscope was used for this project. This was designed for 160-mm tube-length objectives and, in order to allow the modern infinity-corrected TIRFM objective to be used, the Telan (diverging) lens normally fitted in the microscope nosepiece to create an infinity space was removed.

Figure 12B shows a top view of the completed instrument. A  $3 \times 4$  ft optical breadboard is sufficiently large to accommodate the microscope, laser, and other components. To provide working space around the microscope, the laser is mounted to the side with the beam directed by steering mirrors M1 and M2. All optical components, including the laser head, are raised on post mounts to bring the height of the laser beam up to the microscope epifluorescence port. A 30-mW argon ion laser provides more than sufficient power, and a continuously variable neutral density filter in the laser beam allows the intensity at the specimen to be readily controlled. The particular model of laser used provides only a single 488-nm wavelength. If a multiline laser were used a laser line filter would be used to select the desired wavelength. A "ready-made" beam expander provides a fivefold expansion of the laser beam, although this could equally well be constructed (for much lower cost) from two separate lenses. Lens L1 is mounted just before the adjustable mirror (M3) that directs light onto the dichroic mirror, and has a focal length such that the laser beam is focused to a spot at the back of the objective. To achieve correct TIRFM conditions this spot must be positioned near the periphery of the objective back aperture, so the mirror mount holding M3 is mounted, in turn, on a translation stage allowing it to be moved back and forth along the laser beam by a micrometer screw. This motion changes the position at which the reflected beam is incident on the dichroic mirror, and hence the lateral position of the spot at the objective lens.

Initial alignment involves ensuring that the laser beam is centered and parallel to the optical axis of the objective lens. It is easiest to begin with all lenses (beam expander, L1, and the objective) removed. Position the translation stage in the middle of its travel so that it lines up with the dichroic, and place a piece of translucent paper over the empty nosepiece position. Adjust M1 and M2 to center the laser beam on M3, and then adjust the angular position of M3 to center the beam in the nosepiece aperture. Note that rotation of M3 alone changes the angle of the beam going into the microscope, whereas coordinated adjustment of both M2 and M3 can be used to translate the beam (move it from side to side or up and down) without changing its angle. Iterative changes in both these adjustments can then be applied to align the beam so it is both centered and parallel to the optical axis of the microscope, by alternately viewing the position of the beam at the nosepiece and projected onto the ceiling. Once achieved (and this is easier to do in practice than describe!) the beam expander and then L1 can be introduced, with each centered so that the laser beam is not deflected, and the objective lens replaced.

Finally, comes the satisfaction of viewing your first evanescent wave image. A good test specimen is to put a drop of water containing a dilute suspension of  $0.1\text{-}\mu\text{m}$  fluorescent beads on a coverslip. Without moving the translation stage, focus on the bottom of the droplet. You should see a central fluorescent area with stationary beads that have adhered to the coverslip in sharp focus, and other beads that are out of focus to varying extents and are dancing randomly in the water by Brownian motion. Now move the translation stage to bring the laser beam to the periphery of the objective. When total internal reflection is achieved the adherent beads will remain bright, but the motile beads disappear except for brief flashes as they encroach into the evanescent layer near the coverslip.

## Appendix

### *Photonics Publications and Web Sites*

The following journals offer free subscriptions, and provide an excellent guide to recent developments in techniques and equipment.

Photonics Spectra/Biophotonics International	Laser Focus World
Laurin Publications	Penwell Publishing
P.O. Box 4949	P.O. Box 3293
Pittsfield, MA 01202-4949	Northbrook, IL 60065-3293
Phone: (413) 499-0514	FAX: (847) 291-4816
FAX: (413) 442-3180	Website: <a href="http://lfw.pennnet.com/home.cfm/">http://lfw.pennnet.com/home.cfm/</a>
Website: <a href="http://www.photonics.com">www.photonics.com</a>	

The following provides a wide-ranging introduction to all aspects of microscopy, with helpful interactive Java tutorials and an extensive reference list. See especially the review chapter on Optical Microscopy by Davidson and Abramowitz.

Molecular Expressions Microscopy Primer  
Website: <http://micro.magnet.fsu.edu/primer/index.html>

For a clear introduction, starting from the very basics, look for the following:

Patterns in Nature: Light and Optics  
Website: <http://accept.la.asu.edu/PiN/rdg/readings.shtml>

### *Vendors*

The following lists of vendors are not intended to be comprehensive, but primarily include companies from whom I have purchased products. Full listings

are available in the Buyers Guides published by Photonics Spectra and Laser Focus World (see above for addresses), and through the Web sites of these publishers.

### General Optical Suppliers

The companies listed below all sell an extensive range of optical and optomechanical components, and their catalogs are a great resource for information on optical design as well as on specific products.

Coherent Auburn Group  
Catalog Division, A91  
2303 Lindbergh Street  
Auburn, CA 95602-9976  
Toll Free: (800) 343-4912  
Phone: (530) 889-5365  
FAX: (530) 889-5366  
E-mail: info.service@cohr.com  
Website: www.coherentinc.com

Melles Griot  
Photonics Components Div.  
16542 Millikan Avenue  
Irvine, CA 92606  
Toll Free: (800) 835-2626  
Phone: (949) 261-5600  
FAX: (949) 261-7790  
E-mail: sales@irvine.mellesgriot.com  
Website: www.mellesgriot.com

Newport Corp.  
1791 Deere Avenue  
Irvine, CA 92606-4814  
Toll Free: (800) 222-6440  
Phone: (949) 863-3144  
FAX: (949) 253-1680  
E-mail: info@newport.com  
Website: www.newport.com

Thorlabs Inc.  
435 Route 206 North  
Newton NJ 07860  
Phone: (973) 579-7227  
FAX: (973) 300-3600  
Website: www.thorlabs.com

Edmund Industrial Optics  
101 East Gloucester Pike  
Barrington, NJ 08007-1380  
Toll Free: (800) 363-1992  
Phone: (856) 573-6250  
FAX: (856) 573-6295  
E-mail: sales@edmundoptics.com  
Website: www.edmundoptics.com

New Focus, Inc.  
5215 Hellyer Avenue, Suite 100  
San Jose, CA 95138-1001  
Phone: (866) 683-6287  
Fax: (408) 284-4824  
E-mail: contact@newfocus.com  
Website: www.newfocus.com

Spindler & Hoyer Inc.  
459 Fortune Boulevard  
Milford, MA 01757-1745  
Phone: (508) 478-6200  
FAX: (508) 478-5980  
E-mail: info@linos-photonics.com  
Website: www.spindlerhoyer.com

### Optical Filters

These vendors offer specialized filters and dichroic mirrors for fluorescence microscopy. A wide range of colored glass and interference filters is also available from many of the general optical suppliers.

Chroma Technology Corp.  
72 Cotton Mill Hill  
Unit A-9  
Brattleboro, VT 05301  
Toll Free: (800) 824-7662  
Phone: (802) 257-1800  
FAX: (802) 257-9400  
E-mail: sales@chroma.com  
Website: www.chroma.com

Omega Optical Inc.  
210 Main Street  
Brattleboro, VT 05301  
Toll Free: (866) 488-1064  
Phone: (802) 254-2690  
FAX: (802) 254-3937  
E-mail: info@omegafilters.com  
Website: www.omegafilters.com

### Scanners

Both of these companies manufacture a wide range of galvanometer-based scanners. GSI also produce resonant scanners.

Cambridge Technology Inc.  
109 Smith Pl.  
Cambridge, MA 02138  
Phone: (617) 441-0600  
FAX: (617) 497-8800  
E-mail: scanners@camtech.com  
Website: www.camtech.com

GSI Lumonics  
OPTICAL Scanning Products Group Div.  
4E Crosby Drive  
Bedford, MA 01730  
Phone: (781) 275-1300  
FAX: (781) 275-3844  
E-mail: scanning@gsilumonics.com  
Website: www.gsilumonics.com

### Lasers

This list includes only a small fraction of the worldwide laser manufacturers. Some of the general optical suppliers (Melles Griot, Coherent Auburn) sell diode, argon ion, and He-Ne lasers. Evergreen Laser provides efficient, low-cost service and refurbishment for ion lasers.

Coherent Photonics Group  
5100 Patrick Henry Drive  
Santa Clara, CA 95054  
Phone: (408) 764-4983  
FAX: (408) 988-6838  
E-mail: Tech-sales@cohr.com  
Website: www.coherentinc.com

Continuum  
3150 Central Expressway  
Santa Clara, CA 95051  
Phone: (800) 956-7757  
FAX: (408) 727-3550  
E-mail: continuum@ceoi.com  
Website: www.continuumlasers.com

Melles Griot  
Laser Division  
2051 Palomar Airport Rd., #200  
Carlsbad, CA 92009  
Toll Free: (800) 645-2737  
Phone: (760) 438-2131  
FAX: (760) 438-5208  
E-mail: sales@carlsbad.mellesgriot.com  
Website: www.mellesgriot.com

Evergreen Laser Corp.  
9G Commerce Circle  
Durham, CT 06422  
Phone: (860) 349-1797  
FAX: (860) 349-3873  
E-mail: elc@connix.com  
Website: www.evergreenlaser.com

### Detectors

Hamamatsu and Electron Tubes produce a wide range of photomultipliers and accessories. PerkinElmer (previously EG & G, Inc.) supply avalanche photodiode photon-counting modules.

Electron Tubes, Ltd.  
Bury St.  
Ruislip, Middlesex  
HA4 7TA United Kingdom  
Phone: 44-1895-630771  
FAX: 44-1895-635953  
E-mail: info@electron-tubes.co.uk  
Website: www.electron-tubes.co.uk

#### Sales offices:

Electron Tubes Inc.  
100 Forge Way, Unit F  
Rockaway, NJ 07866  
Phone: (800) 521-8382  
FAX: (973) 586-9771  
E-mail: sales@electrontubes.com

PerkinElmer Optoelectronics Headquarters  
2175 Mission College Boulevard  
Santa Clara, CA 95054  
Toll Free: (800) 775-6786  
Phone: (408) 565-0830  
FAX: (408) 565-0703  
E-mail: opto@perkinelmer.com  
Website: www.perkinelmer.com

Spectra-Physics  
335 Terra Bella Avenue  
Mountain View, CA 94043  
Phone: (650) 961-2550  
FAX: (650) 968-5215  
E-mail: sales@splasers.com  
Website: www.spectra-physics.com

Hamamatsu Corp.  
360 Foothill Rd.  
Bridgewater, NJ 08807-0910  
Phone: (908) 231-0960  
FAX: (908) 231-0405  
E-mail: usa@hamamatsu.com  
Website: www.hamamatsu.com  
E-mail: opto@perkinelmer.com

### Flash Lamps and Photolysis Systems

Chadwick-Helmuth Co.  
4601 North Arden Drive  
EI Monte, CA 91731  
Phone: (626) 575-6161  
FAX: (626) 350-4236  
E-mail: chadwick@chadwick-helmuth.com  
Website: www.chadwick-helmuth.com

Cairn Research, Ltd.  
Unit 3G  
Bretons Shipyard Industrial Estate  
Faversham, Kent ME13 7DZ  
United Kingdom  
Tel: +44 0 1 79 559 0140  
FAX: +44 0 1 79 559 0150  
E-mail: sales@cairnweb.com  
Website: www.cairnweb.com

Rapp Optoelektronik  
Gehlenkamp 9a  
D-22559 Hamburg  
Germany  
Phone: +49 40 811330  
FAX: +49 40 814906  
E-mail: info@rapp-opto.com  
Website: www.rapp-opto.com

### Shutters

Vincent Associates  
1255 University Ave.  
Rochester, NY 14607  
Toll Free: (800) 828-6972  
Phone: (716) 473-2232  
FAX: (716) 244-6787  
E-mail: vincent@frontier.net  
Website: www.uniblitz.com

### Electronic Components

Digi-Key Corporation  
701 Brooks Ave. South  
Thief River Falls, MN 56701-0677  
Phone: (800) 344-4539  
FAX: (218) 681-3380  
Website: www.digikey.com

Allied Electronics  
7410 Pebble Drive  
Fort Worth, TX 76118  
Phone: (817) 595-3500  
FAX: (817) 595-6444  
Website: http://www.alliedelec.com

### Imaging Software

Research Systems Inc.  
4990 Pearl East Circle  
Boulder, CO 80301  
Phone: (303) 786-9900  
FAX: (303) 786-9909  
E-mail: info@researchsystems.com  
Website: www.researchsystems.com

Universal Imaging Corp.  
One Ridgewood Place  
Downington, PA 19335  
Phone: (610) 873-5610  
FAX: (610) 873-5499  
E-mail: sales@universal-imaging.com  
Website: www.universal-imaging.com

### Machine Tools and Parts

Rutland and Enco sell tools and accessories, from hand tools to industrial size lathes. Small Parts is a wonderful source for metal and plastic stock, tubing, gears, etc.

Airgas-Rutland Tool  
2225 Workman Mill Rd.  
Whittier, CA 90601-1437  
Phone: (800) 727-9787  
FAX: (800) 444-4787  
Website: [www.rutlandtool.com](http://www.rutlandtool.com)  
[www.airgas.com](http://www.airgas.com)

Enco Manufacturing Co.  
400 Nevada Pacific Highway  
Fernley, NV 89408  
Phone: (800) 873-3626  
Website: [www.use-enco.com](http://www.use-enco.com)

Small Parts Inc.  
13980 N.W. 58<sup>th</sup> Court  
P.O. Box 4650  
Miami Lakes, FL 33014-0650  
Phone: (800) 220-4242  
FAX: (800) 423-9009  
Website: [www.smallparts.com](http://www.smallparts.com)

### Miscellaneous

#### Microscope Immersion Oils

Cargill Laboratories  
55 Commerce Rd.  
Cedar Grove, NJ 07009  
Phone: (973) 239-6633  
FAX: (973) 239-6096  
Website: [www.cargille.com](http://www.cargille.com)

#### Laser Safety Products

Kentek Corp.  
19 Depot St.  
Pittsfield, NH 03263  
Phone: (800) 432-2323  
FAX: (603) 435-7441  
Website: [www.kentek-lasers.com](http://www.kentek-lasers.com)

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